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Status of Zinc in Cow's Milk [1]

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Abstract

A colorimetric method employing dithizone was developed and validated for determination of zinc in milk. The zinc content of cow's milk was found to range from 3 to 6 μg/milliliter. Very little of the zinc in milk is associated with the fat phase, inasmuch as the zinc content of skimmilk is virtually identical to that of the whole milk from which it is derived. An average of about 12% of the total zinc is present in dissolved, ultrafilterable form. The remainder is associated with the caseinate particles. None appears to be associated with the noncasein or whey proteins, inasmuch as the bound zinc is all sedimented with the caseinate under conditions of ultracentrifugation which do not sediment the whey proteins. The casein-bound zinc is all released by acidification to pH 2.0, but only about half of it is removed by dialysis of skimmilk at pH 6.6 or by extraction with dithizone in acetone. Thus, a portion of the zinc appears to be tightly and another loosely bound to constituents of the caseinate complex. The solubilization of bound zinc by adding graded amounts of EDTA to skimmilk further supports the conclusion that zinc is bound in two different manners.

Among the trace elements so far detected in cow's milk, zinc is present in the largest quantity, ranging from 100 to 600 μg/100 ml (2–4, 11, 17), but its partition among the constituents and phases of milk does not seem to have been examined critically and systematically.

Birckner (6) found almost all the zinc in milk to be recoverable in the precipitate obtained by neutralizing the acid serum after removal of casein. Zaykovskii and Chulkov (24) fractionated milk with organic solvents and concluded that zinc (as well as iron and manganese) is present in milk in organic combination. More recently, Imamura et al. (11) determined that about 82% of the zinc in whole milk is found in skimmilk and 76% in acid whey. Remarkably, from these results they classified zinc among those elements adsorbed by fat globules.

The present paper reports some analyses of the distribution of zinc in milk as obtained by techniques of centrifugation, ultrafiltration, dialysis, and extraction.

Methods

The dithizone spectrophotometric method described by Sandell (19) for estimation of trace concentrations of zinc was used in these investigations.

Reagents

Dithizone. 0.0005% in A. R. CCl_4. Reagent-grade dithizone (G. Frederick Smith Company) was used.

Acetate buffer. pH 4.75. Mix equal volumes of 2 M sodium acetate and 2 N acetic acid and remove reacting heavy metals by shaking with 0.01% dithizone solution in CCl_4. Filter through a small quantitative filter paper to remove CCl_4 droplets.

Sodium thiosulfate. Dissolve 25 g of $Na_2S_2O_3 \cdot 5\ H_2O$ in 100 ml of deionized distilled water.

Standard zinc solution. Dissolve 0.1 g of zinc (C.P.) in 10 ml of HCl (sp gr 1.20), evaporate to dryness, dissolve in water, and dilute to one liter. One milliliter of this solution contains 100 μg of zinc.

Preparation of samples. Incinerate a 10-ml aliquot of the sample in a platinum dish at 600 C in a muffle furnace overnight. Moisten with a little distilled water, dissolve in 1 ml of 1 N HCl, transfer quantitatively to a 10-m volumetric flask, and make to volume.

Procedure. Pipette 1 ml of ash solution and 4 ml of water into a 25-ml separatory funnel. The amount of zinc present should lie between 1 and 7 μg. Add 5 ml of acetate buffer (pH 4.75) and 1 ml of thiosulfate solution, followed by 10 ml of 0.0005% dithizone solution. Stopper the funnel, shake vigorously for about 2 min, and run the clear CCl_4 extract into a cuvette. Measure optical densities by Beckman Model B spectrophotometer at wave lengths of 620 and 530 mμ, employing A. R. CCl_4 as a blank. The amount of zinc present is obtained from a standard curve (Figure 1) prepared by plotting difference in absorbance

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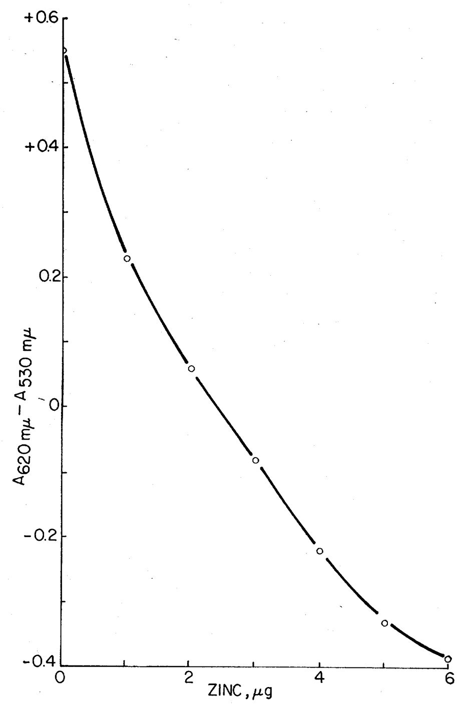

FIG. 1. Standard curve dithizone method.

at the two wave lengths (A620–A530) against zinc content of standard solutions.

This method is similar to that used by Vallee and Gibson (22) for samples of blood and tissue. Tartrate and cyanide, however, are omitted, since possible interfering metals such as copper, iron, cobalt, and nickel are present in much smaller concentrations than zinc. The standard curve could be prepared, like that of Vallee and Gibson, by plotting zinc concentration against A530–A620/R, where R is A620/A530 for dithizone itself. This method did not seem, however, to offer any particular advantage over the plot of A620–A530. Fractions of milk were prepared in the following ways:

1. *Skimmilk*: Whole milk at 40 C was centrifuged in an International no. 2 centrifuge at 2,500 rpm [ca. 500 $\times$ *g* (max)] for 25 min and cooled to 5 C for 1 hr. The cream layer was punctured and the skimmilk removed by siphoning.

2. *Pressure ultrafiltrate*. Skimmilk was ultrafiltered through washed cellophane casing supported by a stainless steel screen (20) at room temperature and a pressure 10 psi from a nitrogen tank or compressed airline. The volume of ultrafiltrate collected did not exceed 10 ml per 100 ml of skimmilk.

3. *Equilibrium dialysate*. A volume of water in a cellophane casing was dialyzed against several changes of whole or skimmilk at 0–5 C.

4. *Centrifuge whey*. Skimmilk was centrifuged for 3 hr at 5 C at a speed of 40,000 rpm [144,880 $\times$ *g* (max)] in a Type 40 rotor in a Spinco Model L ultracentrifuge or subjected to similar centrifuging in an International B–35 centrifuge.

5. *Rennet whey*. One hundred milliliters of skimmilk at 35 C was treated with 0.1 ml of commercial rennet extract. After 30 min the curd was cut into small cubes. The whey exuded was centrifuged at 2,000 rpm [ca. 1,200 $\times$ *g* (max)] for 10 min in the International no. 2 centrifuge.

6. *Isoelectric whey*. Skimmilk at room temperature was acidified to pH 4.6 with 1 N HCl and centrifuged for 10 min at 2,000 rpm [ca. 1,200 $\times$ *g* (max)] in the International no. 2 centrifuge.

7. *Colloidal-phosphate-free milk*. Skimmilk was acidified to pH 4.9 at 0–5 C and dialyzed vs. skimmilk or whole milk for 48–96 hr at 0–5 C, as described by Pyne and McGann (18).

Milk specimens were concentrated in glass vacuum rotatory evaporator at a temperature below 50 C. Heat-coagulation time was determined by rocking 1-ml samples in sealed Pyrex tubes in a silicone oil bath at 135 C until the appearance of a visible coagulum.

Results

Precision and accuracy of the method. Duplicate determinations on 16 samples of skimmilk ash ranging from 3.10 to 6.00 μg Zn per milliliter gave a standard deviation of 0.079 μg/milliliter. For duplicate determinations on 16 samples of ashed ultrafiltrate ranging from 0.14 to 1.20 μg/milliliter, the standard deviation was 0.023 μg/milliliter. It is emphasized that these are estimates of the precision of the dithizone colorimetric method; they do not include errors of the sampling, incinerating, or ultrafiltration procedures.

To check the accuracy of the method, recovery of added zinc was determined. One or two micrograms of zinc was added as a solution of the chloride to 1 ml of each of eight samples of skimmilk and four samples of pressure ultrafiltrate before ashing (Table 1). Recoveries of 90–100% from the skimmilk and 88.5–100% from the ultrafiltrate seem reasonably satisfactory. In a further check of recovery, 1 to 5 μg of zinc as the chloride was added to 5-ml aliquots of the artificial salt solution described by Jenness and Koops (12); recoveries of 96–115% were obtained (Table 2).

To determine if iron and copper present in milk interfere in the estimation of zinc by the

TABLE 1
Recovery of zinc added to milk or its pressure ultrafiltrate

Sample no.	Zinc already present	Zn added	Zn estimated in mixture	Recovery of added Zn
	($\mu g/ml$)			(%)
Bulk milk samples				
1	3.58	1.00	4.58	100.0
2	3.58	2.00	5.55	98.5
3	3.80	1.00	4.70	90.0
4	3.30	1.00	4.30	100.0
5	4.70	1.00	5.70	100.0
6	6.00	1.00	6.95	95.0
7	4.00	1.00	5.00	100.0
8	3.68	1.00	4.65	97.0
Average	4.08	1.13	5.19	98.2
Pressure ultrafiltrate of bulk milk samples				
1	0.84	2.00	2.61	88.5
2	0.57	1.00	1.57	100.0
3	0.75	1.00	1.65	90.0
4	0.60	1.00	1.60	100.0
Average	0.69	1.25	1.88	95.2

TABLE 2
Recovery of zinc added to artificial milk salt solution

Zn added	Zn estimated	Recovery of added Zn
(μg)		(%)
0	0	......
1	1.15	115
2	2.30	115
3	2.95	98
4	3.85	96
5	4.95	99

method employed, 1 to 5 μg of iron as $FeSO_4$ and 1 to 5 μg of copper as $CuSO_4$ were added to 5-ml portions of the artificial salt solution described by Jenness and Koops (12). The presence of either of these two metals, at concentrations far greater than actually present in milk, did not interfere at all in the estimation of zinc.

Content and distribution of zinc in milk. Since it was desired, if possible to use milk samples obtained by machine milking, a comparison was first made of the zinc content of hand- and machine-milked samples. With each of three cows machine milking was interrupted at about midpoint and a small amount of milk drawn by hand into a plastic beaker. Machine milking was then completed and the contents of the milking-machine pail sampled. Zinc contents of skimmilks prepared from hand- and machine-milk samples were identical (Table 3). In all subsequent experiments, specimens from individual cows were obtained by sampling from the milking machine at the end of milking. Bulk milk samples were obtained from the bulk tank at the University dairy barn.

TABLE 3
Zinc contents of skimmilk of various cows milked by hand and by machine

Cow no.	Total zinc: Hand-milked sample	Total zinc: Machine-milked sample	Pressure ultrafiltrate: Hand-milked sample	Pressure ultrafiltrate: Machine-milked sample
	($\mu g/ml$)			
1	3.00	3.00	0.43	0.50
2	3.15	3.20	......	0.30
3	3.20	3.20	0.30	0.27

TABLE 4
Comparison of zinc contents of whole and skimmilk

Sample no.	Zn in: Whole milk	Zn in: Skimmilk
	($\mu g/ml$)	
1	3.30	3.25
2	3.70	3.50
3	3.25	3.20
4	3.85	3.90
5	3.70	3.85
6	4.00	4.00

The distribution of zinc between fat globules and the fat-free plasma was determined by analysis of six samples of whole milk and the corresponding skimmilks (Table 4). The zinc content of skimmilk is very close to that of whole milk and the data give no clear-cut evidence that any zinc is bound by fat globules.

The distribution of zinc between the dissolved and collodial states for 18 samples of milk from the bulk tank and 14 samples from 14 individual cows of different breeds and lactation periods, obtained on different days (March–July, 1965) from the University dairy herd, is recorded in Table 5. The range of variations for total zinc in bulk samples is 3.1 to 6.0 μg/milliliter, with an average of 3.84 μg/milliliter; whereas, the corresponding range for individual samples is 3.2–6.3 μg/milliliter, the average being 4.78 μg/milliliter.

Zinc present in the dissolved state, estimated from pressure ultrafiltrates of skimmilk, represents 3.2 to 22.9% of the total zinc in bulk samples and 2.3 to 15.9% in the individual samples, the averages being 12.06 and 11.35%, respectively. The colloid-bound zinc, therefore, constitutes a major portion, averaging 88% of the total zinc in skimmilk.

To determine the fraction of zinc, if any,

TABLE 5

Distribution of zinc in milk between dissolved and colloidal phases

Sample no.	Zinc: Total	Zinc: Dissolved	Zinc: Colloidal[a]	Zinc in dissolved state
	(μg/ml)			(%)
Bulk milk from University herd				
1	3.87	0.46	3.41	11.88
2	3.60	0.42	3.18	11.66
3	3.71	0.42	3.29	11.32
4	3.55	0.41	3.14	11.55
5	3.62	0.41	3.21	11.32
6	3.80	0.78	3.02	20.52
7	3.58	0.41	3.17	11.45
8	3.75	0.86	2.89	22.93
9	4.70	0.57	4.13	12.12
10	6.00	1.20	4.80	20.00
11	3.10	0.23	2.87	7.42
12	3.60	0.32	3.28	8.88
13	3.70	0.42	3.28	11.35
14	3.75	0.23	3.52	6.13
15	4.00	0.13	3.87	3.25
16	4.00	0.35	3.65	8.75
17	3.68	0.30	3.38	8.28
18	3.20	0.44	2.76	13.75
Average	3.84	0.46	3.38	12.06
Individual milk samples from University herd				
1	6.00	0.85	5.15	14.16
2	5.50	0.72	4.78	13.09
3	4.70	0.75	3.95	15.95
4	6.00	0.83	5.17	13.83
5	3.20	0.30	2.90	9.37
6	6.30	0.80	5.50	12.69
7	5.50	0.60	4.90	10.91
8	5.60	0.70	4.90	12.50
9	3.20	0.46	2.74	14.37
10	3.70	0.33	3.37	8.92
11	4.20	0.10	4.10	2.38
12	4.65	0.20	4.45	4.30
13	4.50	0.53	3.97	11.77
14	3.90	0.43	3.47	11.02
Average	4.78	0.54	4.24	11.35

[a] Calculated by difference.

bound to whey proteins, three samples of skimmilk were centrifuged at 40,000 rpm for 3 hr and the clear supernatants, containing whey proteins, dissolved salts and lactose analyzed for zinc content. The results, in comparison to their pressure ultrafiltrates, which contain only dissolved salts and lactose, are given in Table 6. A close agreement between the zinc contents of the ultrafiltrate and the centrifuge supernatant indicates the absence of any zinc bound to whey proteins.

Three samples of skimmilk were heated at 90 C for 30 min in 30-ml stainless steel tubes cooled immediately to about 5 C and ultrafiltered. Results given in Table 7 show a small transference of dissolved zinc to the colloidal phase on heat treatment.

Distribution of added zinc. Amounts of zinc ranging from 1 to 5 μg/milliliter were added as a solution of $ZnCl_2$ to skimmilk, rennet whey, and colloidal-phosphate-free skimmilk and its distribution determined by ultrafiltration, equilibrium dialysis, and ultracentrifugation. As shown in Figure 2, most of the zinc added to skimmilk is bound in a nonultrafilterable, centrifugable form.

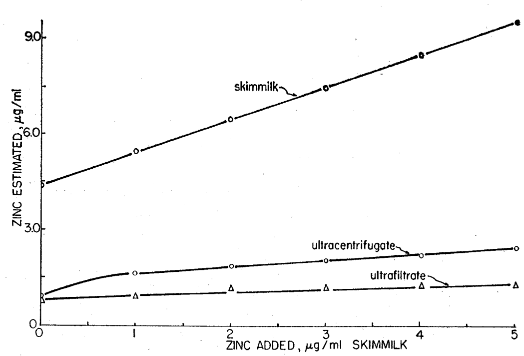

FIG. 2. Distribution of added zinc in skimmilk.

Figure 3 shows the effect of adding zinc to rennet whey. Zinc was not removed by ultracentrifugation, but since the protein is not sedimented this treatment does not indicate whether zinc is bound by whey proteins. Equilibrium dialysis of water against large volumes of whey to which zinc had been added showed the zinc to be entirely diffusible. Results of

TABLE 6

Zinc in milk fractions

Fraction	Contents[a]	Zinc: Sample I	II	III
		(μg/ml)		
Skimmilk	C + W + S	4.00	3.70	3.90
Ultracentrifugate	W + S	0.46	0.52	0.55
Rennet whey	W + S	0.40	0.48	0.47
Isoelectric whey	W + S	2.10	2.00	2.30
Ultrafiltrate	S	0.42	0.52	0.60
Equilibrium dialysate	S	0.52	0.43	0.60

[a] C = Casein. W = Whey protein. S = Sugars, salts, and other dissolved substances.

TABLE 7
Effect of heating skimmilk on zinc distribution

	Sample I		Sample II		Sample III	
Zinc	Raw	Heated 90 C–30 min	Raw	Heated 90 C–30 min	Raw	Heated 90 C–30 min
Total *(μg/ml)*	3.7	3.9	3.6	3.7	4.3	4.4
Ultrafilterable *(μg/ml)*	0.8	0.5	0.4	0.3	0.5	0.3
Colloid-bound *(μg/ml)*	2.9	3.4	3.2	3.4	3.8	4.1

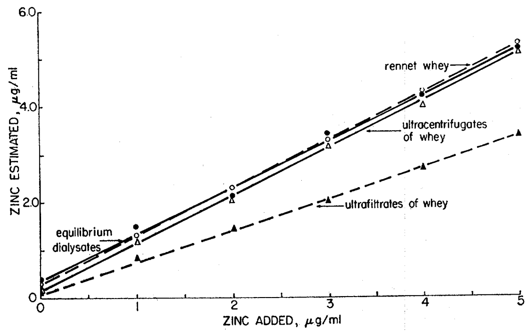

Fig. 3. Distribution of added zinc in rennet whey.

pressure ultrafiltration, on the other hand, seem to indicate some binding, but perhaps equilibrium is not attained or zinc is hindered in passage through the membrane in this procedure. A very much greater area of cellophane is exposed per unit volume of whey in the pressure ultrafiltration than in equilibrium dialysis, and perhaps binding of zinc by the membrane occurs, especially since the system is devoid of caseinate which would offer competing binding sites.

Zinc added to colloidal-phosphate-free milk is bound almost entirely, as shown in Figure 4.

The effect of adding 45 μg of zinc per milliliter as $ZnSO_4$ on the heat stability at 135 C

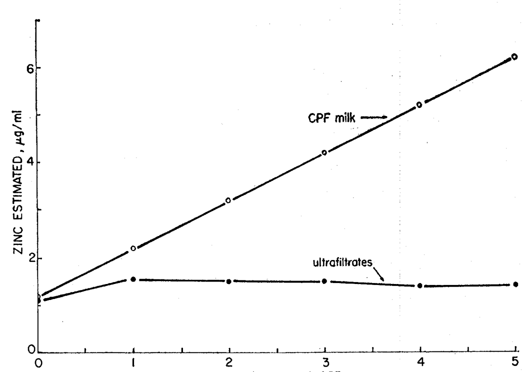

Fig. 4. Distribution of added zinc in colloidal phosphate-free (CPF) milk.

was determined. As shown in Table 8, the heat stability of either the unconcentrated or 2:1 concentrated skimmilk is not greatly affected by this large increase in zinc content.

TABLE 8
Effect of added zinc on heat coagulation

	Sample A	Sample B
Zinc added *(μg/ml)*	0	45
pH	6.68	6.66
Coag. time (min) at 135 C		
Raw		
Unconcentrated	30.2	25.0
Concentrated 2:1	5.0	3.6
Heated 90 C − 10 min		
Unconcentrated	26.1	26.0
Concentrated 2:1	13.1	9.1

Solubilization of bound zinc. A number of methods were used in attempts to release the bound zinc and thereby, perhaps, to find clues as to the manner in which it is bound.

Exhaustive dialysis of skimmilk against zinc-free phosphate buffer at pH 6.6 and ionic strength — 0.1 resulted in release of about 50% of the total zinc present (Figure 5). About 1.7 μg of zinc per milliliter appears to be very strongly bound and only very slowly solubilized by this dialysis procedure. Likewise, added zinc seems to be only slowly dialyzable.

The effect of adding ethylenediamine tetraacetate (EDTA) was next studied. Graded amounts of Na_2EDTA were added to separate aliquots of a lot of skimmilk and the pH of each aliquot adjusted to 6.6 by appropriate addition of NaOH. The amount of dilution was kept constant at 2 ml/100 ml by adding water as needed. Ultrafilterable zinc was determined in each prepared sample, as shown in Figure 6. Addition of 2 μmoles of EDTA per milliliter, equivalent to 40 times the zinc concentration, failed to solubilize about 1.6 μg of zinc per milliliter.

In another experiment, 35 μmoles per milliliter of EDTA was added as a dry mixture of the disodium and tetrasodium salts, in proportions found not to change the pH of the milk.

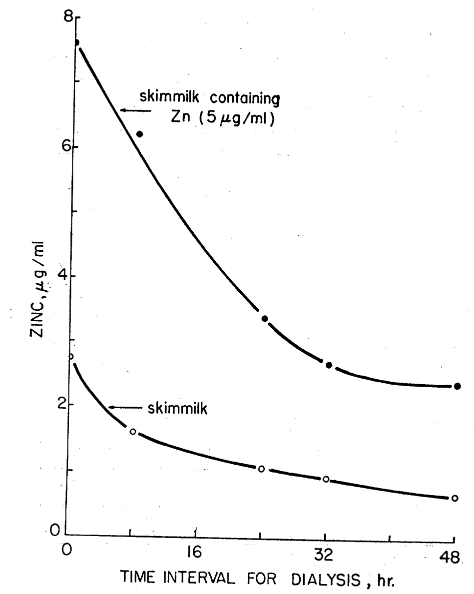

FIG. 5. Effect of dialysis of skimmilk vs. phosphate buffer (pH 6.60) on zinc content.

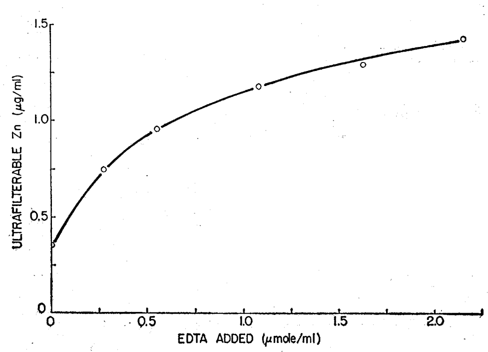

FIG. 6. Effect of added EDTA (2 μmole/milliliter) on ultrafilterable zinc.

This quantity of EDTA is sufficient to bind all of the calcium and magnesium in the milk and thus to disaggregate the caseinate-phosphate micelles. All of the zinc is rendered ultrafilterable by this treatment. The influence of adding graded amounts of EDTA up to 35 μmole/milliliter at constant pH was next studied. As shown in Figure 7, there is a sharp inflection in the curve, which further implies that zinc is bound in two different manners.

The extractability of zinc by dithizone was determined with a method used by Vikbladh (23) for blood serum. Five-milliliter aliquots of skimmilk were shaken in a centrifuge tube with two successive 45-ml portions of 0.015% dithizone in acetone, the precipitate being centrifuged down each time. That this is an ex-

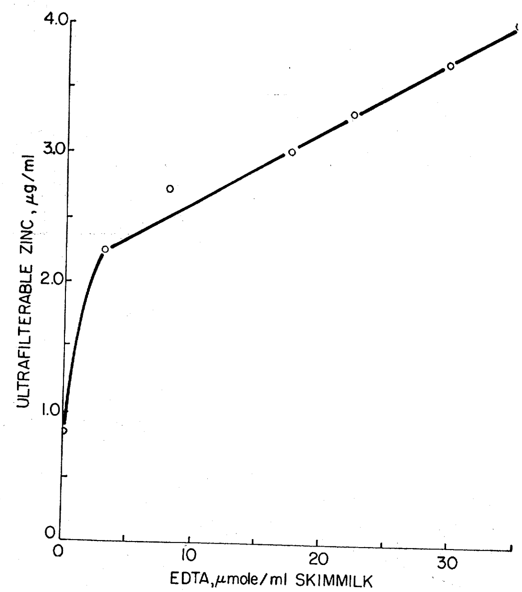

FIG. 7. Effect of added EDTA (30 μmole/milliliter) on ultrafilterable zinc.

cess of dithizone is attested to by the fact that the solution remained green on the second extraction. The precipitate was then washed with two 45-ml portions of acetone, ashed, and analyzed for zinc. As shown in Table 9, the

TABLE 9

Release of bound zinc in skimmilk on shaking with dithizone in acetone

Sample	Total Zn	Total Zn after treatment with dithizone in acetone	Loosely bound Zn
	(μg/ml)		(%)
1	3.70	2.25	39.2
2	3.10	1.75	43.5

amount of zinc not extracted by this treatment amounted to 1.75–2.25 μg per milliliter.

The effect of lowering pH on the content of ultrafilterable zinc in skimmilk was next studied. Aliquots were adjusted to pH's down to pH 2.0, with 10 N HCl, the dilution being kept constant at 2 ml/100 ml by appropriate addition of water. Figure 8 shows that the release of bound zinc is about complete at pH 2 to 3.

Discussion

The analyses reported herein indicate zinc contents of 3.1 to 6.0 μg per milliliter of milk, similar to those reported in the literature. Only

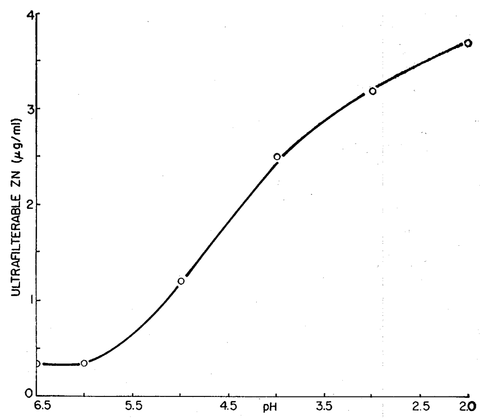

FIG. 8. Effect of pH on ultrafilterable zinc.

a small amount of the zinc in milk appears to be associated with the fat globules. The difference in zinc contents of whole and skimmilk is within the limits of error of the analytical method used. This finding agrees with the report of Herald et al. (10) of a zinc content of 20 μg/gram of fat gobue membrane protein. Assuming that there is 0.5 g of membrane protein per 100 g of fat, then 4% fat milk would contain only $\frac{20 \times 0.5 \times .04}{100} = 0.004$ μg zinc/ml bound in the fat globule membrane.

Imamura et al. (11) reported somewhat greater proportions (13, 18, and 24%) of the total zinc in milk in the fat phase, on the basis of analyses of three samples of whole milk and the corresponding skimmilks. The highest value in the present work is about 6% (Table 4). The reason for this discrepancy is not evident, but in any case Imamura's classification of zinc as a mineral adsorbed by fat globules is somewhat misleading. The distribution of zinc is in marked contrast to that of copper or iron, large proportions of which are bound to the fat globules (11, 14).

The ultrafilterable zinc evidently exists partly or largely in the form of undissociated complexes, since it is not completely extractable by shaking milk ultrafiltrate with a solution of dithizone in CCl_4. It is reasonable to suppose that zinc may be present as a complex with citrate, inasmuch as zinc is not readily extracted by dithizone from citrate buffers at pH 6.6 (7).

The nonultrafilterable zinc of milk seems to be bound by one or more of the constituents of casein. The present analyses give no clue as to whether any of the individual casein components preferentially bind zinc. Allred et al. (1) have shown that α-casein (largely α_{s1} and κ-caseins) binds zinc at pH's between 3.7 and 5.3, a finding consistent with those reported herein. Apparently, the whey proteins do not bind any significant amount of zinc, inasmuch as the zinc content of rennet whey which contains them is approximately equal to that of ultrafiltrate which contains no whey protein.

The bound zinc of milk does not all appear to be held with equal tenacity by its ligands. Vikbladh (23) applied the technique of extracting with dithizone in acetone to blood serum and found that about 67% of the zinc could be extracted (loosely bound) and the remainder (33%) could not (tightly bound). Application of Vikbladh's technique to skimmilk revealed that about 1.5–1.8 μg of zinc per milliliter could not be extracted and might, thus, be termed tightly bound. It is of interest that approximately this same quantity of zinc is resistant to solubilization by adding EDTA or by dialysis against zinc-free phosphate buffer. Therefore, it appears that zinc is present in milk in at least three states, as follows: a) ions and complexes in solution, 0.5 μg/milliliter; b) loosely bound complexes with casein, 1.7 μg/milliliter; c) tightly bound complexes with casein, 1.8 μg/milliliter. The actual manner in which the zinc is bound is not understood. That it is bound by casein or fractions thereof or materials associated therewith and is not bound by whey protein suggests that certain specific linkages may be involved. It is possible that part of the bound zinc is present in one or more enzymes associated with the caseinate in milk. Several zinc metalloenzymes have been described and characterized. These are carbonic anhydrase from bovine erythrocytes, alcohol dehydrogenase from yeast and equine liver, glutamic dehydrogenase of rabbit skeletal muscle, carboxypeptidase from bovine pancreas, and alkaline phosphatase from porcine kidney and *E. coli.* (21). Milk contains considerable alkaline phosphatase (13) which has been suggested to be a zinc enzyme (16). Rigorous proof seems to be lacking, however, and in any event the enzyme is largely associated with fat globules rather than caseinate in milk (13).

Another possibility is that one or more of the caseins themselves contain specific groupings having high affinity for zinc. Such structures as the ester phosphate groups of casein, a pair of imidazole groups as in insulin (9), or a carboxyl and thiol in proper juxtaposition as in carboxy-peptidase A (8) are by no means inconceivable. Binding through thiol would be limited to κ-casein, considered by Beeby (5) to contain such groups, or to minor casein fractions. α_{s1} and β-caseins do not appear to contain free -SH groups.

It is also conceivable that a portion of the zinc is bound by the inorganic colloidal phosphate. The evidence suggests that the easily removable bound zinc resides on the surface of the caseinate-phosphate micelles and that added zinc is bound also on the micellar surface. Only by disruption of the micelles can all of the zinc be extracted with EDTA.

Leviton and Pallansch (15) indicated that added zinc sulfate improved storage stability of H.T.S.T. sterilized concentrated milk, but added zinc seems to be without effect on heat coagulation.

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